Variation in K-*ras* Codon 12 Point Mutation Rate with Histological Atypia within Individual Colorectal Tumors

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To elucidate genetic alteration in relation to morphology and also to confirm more directly the proposed adenoma-carcinoma sequence, we analyzed thirty-eight colorectal "cancer in adenoma" lesions exhibiting areas of different atypia, in terms of K-*ras* codon 12 point mutation. The mutation incidence was 26.3% (10/38) for all cancerous areas. Well-differentiated and very well-differentiated carcinoma exhibited values of 17.6% (3/17) and 30.4% (7/23), respectively (statistically not significant). Positive cases of adenoma with severe atypia and adenoma with moderate or slight atypia were 26.7% (8/30) and 8.3% (3/36) respectively (statistically significant). Thus, K-*ras* point mutation, as indicated previously, may play an important role in the early stages of colorectal tumorigenesis. As for the nature of the mutation, GGT(Gly) to GAT(Asp) was the most frequent (80%). Eight cases had mutations concurrently in different areas of the same tumor and in all of these the mutation was homogeneous (6 cases to GAT, 1 case to TGT and 1 case to GTT). This provides genetic support for the "adenoma-carcinoma sequence" theory proposed on the basis of morphological considerations. All lesions with a mutation were of polyploid type, and no mutation was found in the flat type.

Key words: K-*ras* — Large intestinal neoplasm — Tumorigenesis

In colorectal tumors, several gene alterations involving ras,[1-10] p53,[11] DCC,[12] MCC,[13] or APC[14-17] have been reported. It is generally supposed that multi-step gene alterations contribute to colorectal carcinogenesis. The fact that colorectal tumors, particularly those limited to the mucosa or submucosa, frequently comprise several different atypias including carcinomatous elements provides a useful model for comparative gene analysis. Further, the possibility of assessing intra-tumor variation allows a direct check of the "adenoma-carcinoma sequence" theory suggested on the basis of histological considerations. Recently, Yanagisawa et al.[18] succeeded in detecting variation in K-*ras* codon 12 point mutation in mucinous cystic neoplasms of the pancreas relative to histological atypias within the same tumor by using small samples of tissue from the slide glasses. In the present study, the same approach was adopted to analyze colorectal lesions of "cancer in adenoma" in order to examine whether a genetic basis may exist for the adenoma-carcinoma sequence.

MATERIALS AND METHODS

DNA extraction and amplification   Thirty-eight colorectal lesions of "cancer in adenoma" (35 polyploid tumors and 3 flat tumors) were analyzed. All of them were endoscopically resected in the Cancer Institute Hospital or the Hiratsuka Icho Hospital. Biopsy samples from obviously advanced cancers were also analyzed.

DNA extraction from paraffin sections was performed according to the procedure described previously.[18] Serial sections of 4 μm, 10 μm, 10 μm, 4 μm were made from paraffin blocks and attached to the slides. The two 4 μm sections were stained with hematoxylin and eosin, and examined for the presence of the following four area types: cancer (well-differentiated adenocarcinoma, WDC or very well-differentiated adenocarcinoma, VWDC), adenoma with severe atypia; adenoma with moderate or slight atypia; and normal mucosa. Basically, only one sample was taken from each area. Differential diagnosis between WDC and VWDC was made in terms of structural and cellular atypia (Figs. 1 and 2). All adenomatous areas examined were of tubular type and their degrees of atypia were determined according to the criteria of the Japanese Research Society for Cancer of Colon and Rectum (Figs. 3 and 4). Normal mucosa was taken from areas just adjacent to the tumor.

Limited areas, each comprising 100–1000 cells, were cut out under a stereomicroscope from one of the two 10 μm sections, and stained with hematoxylin only (the second one was used as a spare for the study).

Tissue samples were again deparaffinized with xylene, rinsed with ethanol, completely dried, digested with proteinase K (500 μg/ml–1 mg/ml) and boiled prior to performance of the polymerase chain reaction (PCR) procedure (40 cycles).

DNA corresponding to the 12th codon of c-K-*ras* was amplified by the PCR technique essentially according to

the method described by Saiki *et al.*[19] and Wright and Manos[20] using a PCR machine and kit (Perkin Elmer Cetus). Primers used for amplification were TTGTTG-GATCATATTCGTCC and GGCCTGCTGAAAATG-ACTGA, which yielded 118-bp amplified DNA fragments around codon 12 of K-*ras* gene.

DNA probes and hybridization A 2 μl sample of each amplified DNA was spotted onto Hybond-N$^+$ filters (Amersham) and fixed by the alkali procedure. Oligonucleotide hybridization was performed as described by Verlaan-de Vries *et al.*[21] A series of specific synthetic 19mer anti-sense single-stranded DNA probes, corresponding to possible point mutations at codon 12, were end-labeled using [γ-^{32}P]ATP polynucleotide kinase (Toyobo).

Final washing was done with 3 M tetramethylammonium chloride, 50 mM Tris/HCl (pH 7.5), 2 mM EDTA and 0.1% sodium dodecyl sulfate at 59°C.

RESULTS

Incidence of K-*ras* codon 12 point mutations relative to histological atypia (Table I) K-*ras* codon 12 point mutations were detected in ten of 40 cancerous areas from 38 lesions of "cancer in adenoma" (35 of polypoid type and 3 of flat type). In two of the flat types, 2 different cancerous areas were examined, but none was associated with a mutation. The mutation incidence for all 38 cases was 26.3% (10/38). Mutations were seen only in the polypoid type at an incidence of 28.6% (10/35).

Table I. Incidences of K-*ras* Codon 12 Point Mutation Relative to Histological Atypia in 38 Colorectal Tumors

Histological atypia		Gross type of polyp		Total
		Polypoid type	Flat type	
Cancer	Well diff.	3/15 (20.0%)	0/2 (0%)	3/17 (17.6%)
	Very well diff.	7/20 (35.0%)	0/3 (0%)	7/23 (30.4%)
Cancer (Total)		10/35 (28.6%)	0/5 (0%)	10/40 (25.0%)
			0/3 (0%)[b]	10/38 (26.3%)[b]
Adenoma	Severe atypia	8/27 (29.6%)⎤*	0/3 (0%)	8/30 (26.7%)⎤*
	Mod/sl[a] atypia	3/35 (8.6%)⎦	0/1 (0%)	3/36 (8.3%)⎦
Normal		0/35 (0%)	0/3 (0%)	0/38 (0%)
Advanced cancer	Well diff.	3/15 (20.0%)		5/19 (26.3%)
	Very well diff.	2/4 (50.0%)		

a) Mod/sl: moderate or slight atypia.   *b*) Incidences for all cases.
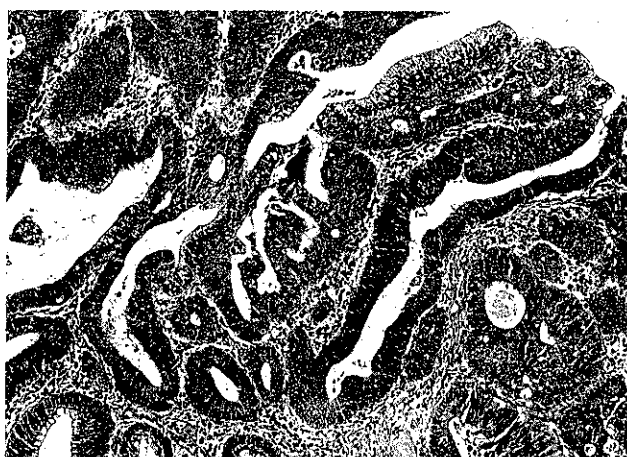*$P<0.05$.

Fig. 1. Well-differentiated adenocarcinoma (WDC). Structural atypias such as a cribriform pattern, back-to-back appearance, and irregularity in gland size and shape are prominent, together with cellular atypias such as a high nucleocytoplasmic ratio, pleomorphism in nuclear size and shape, pseudostratification or loss of polarity of the nucleus.

Fig. 2. Very well-differentiated adenocarcinoma (VWDC). Patterns for WDC are less prominent: tubular or papillary structures are simpler, more regular and more homogeneously distributed. Further, rounded or oval nuclei are mostly located near the basement membrane.

When the carcinomas were divided into two groups, WDC and VWDC (Figs. 1 and 2), the total incidences were 17.6% (3/17) and 30.4% (7/23) respectively, and for the polypoid type described above, 20.0% (3/15) and 35.0% (7/20). None of these differences was statistically significant. As for the advanced cancer group, the overall incidence was 26.3% (5/19) and the incidences for WDC and VWDC were 20.0% (3/15) and 50.0% (2/4), respectively, the difference again not being significant.

In contrast, the incidences for adenomatous areas with severe atypia or with moderate or slight atypia (Figs. 3 and 4) were significantly different at 26.7% (8/30) and 8.3% (3/36), respectively. As with the cancerous areas, mutations were only seen in lesions of polypoid type, the respective incidences being 29.6% (8/27) and 8.6% (3/35). No mutation was detected in normal mucosa samples.
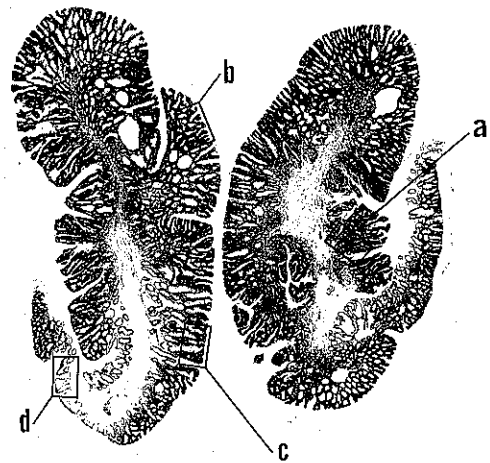

Fig. 3. An example of "cancer in adenoma" of the colon (case 7). Loupe view.
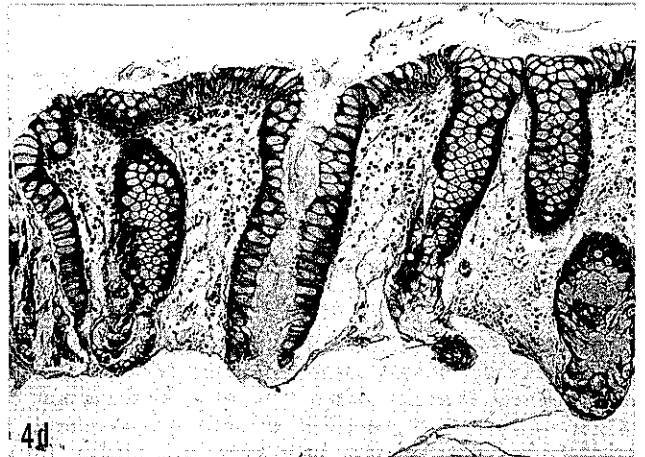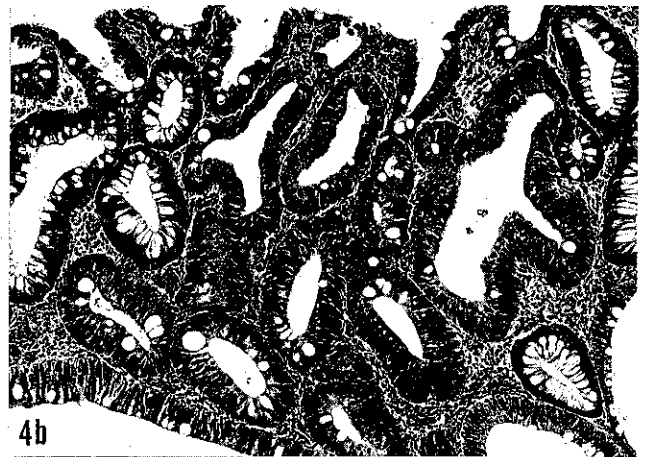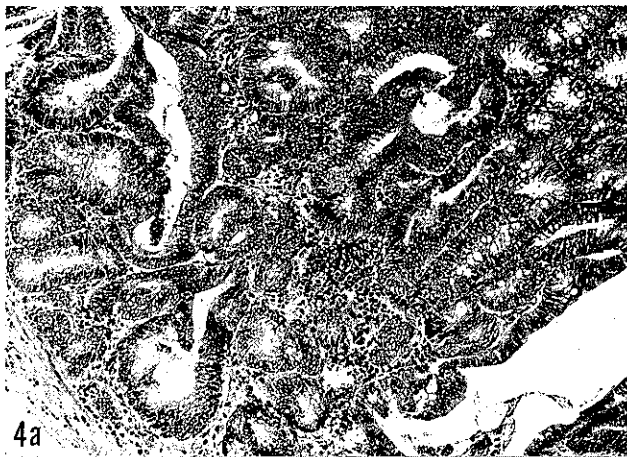

Fig. 4. High magnification views of the respective boxed areas (a–d) in Fig. 3. a, VWDC; b, adenoma with severe atypia; c, adenoma with slight atypia; d, normal mucosa.

Table II. Colorectal Tumors with a K-*ras* Codon 12 Point Mutation

Case	Sex/Age	Site	Shape	Size (mm)	Cancer		Adenoma		Normal
					Well-diff.	Very Well-diff.	Severe atypia	Mod/sl atypia	
1	M/59	R-S	P	10	TGT	·	TGT	—	—
2	M/72	R-S	P	11	·	GTT	GTT	—	—
3	M/59	Asc	P	7	·	GAT	—	—	—
4	M/59	Asc	P	15	·	GAT	GAT	—	—
5	F/54	R-S	P	20	·	GAT	GAT	GAT	—
6	M/51	R-S	P	30	·	GAT	GAT	—	—
7	F/?	Asc	P	7	·	GAT	GAT	—	—
8	M/59	R-S	P	15	GAT	·	GAT	GAT	—
9	M/50	R-S	P	12	GAT	·	GAT	GAT	—
10	M/54	R-S	P	15	·	GAT	—	—	—

R-S: Rectosigmoid colon. P: Polypoid. Asc: Ascending colon. Mod/sl: moderate or slight atypia. ·: Not examined. —: No mutation.
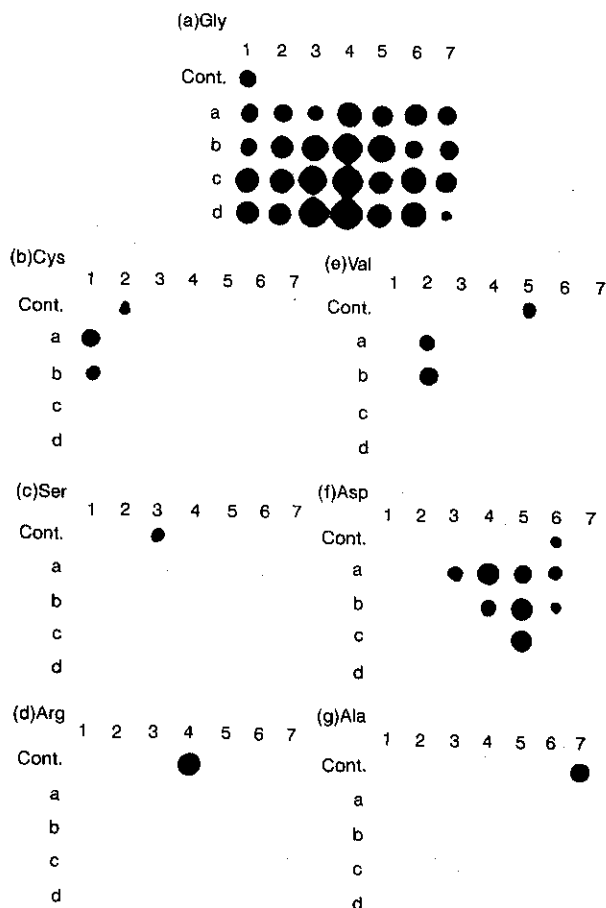
Nature of the K-*ras* codon 12 point mutation in 10 colorectal tumors (Table II and Fig. 5) The K-*ras* codon 12 point mutation resulted in a transition from GGT to GAT in eight cases, and to TGT and GTT in one case each. Eight out of the 10 showed the mutation simultaneously in different areas within the same tumor. For example, case 7 demonstrated the same mutation to GAT in both areas with cancer and with severe atypia and case 1 similarly exhibited consistent mutation to TGT. Thus, in all cases the mutations demonstrated were homogeneous.

DISCUSSION

Many researchers have reported the presence of K-*ras* point mutations in colorectal cancers, mostly in advanced lesions. Incidences were generally 25–40% with respect to mutation at codon 12: 33% (9/27) was found by Bos et al.,[1] 39.4% (26/66) by Forrester et al.,[2] 32.6% (30/92) by Vogelstein et al.,[3] 29.0% (9/31) by Salhab et al.,[4] and 26.4% by Ando et al.[6] Miyaki et al.[5] found 21.7% (5/23) of mucosal carcinomas including "cancer

in adenoma" to be positive. Our results for advanced cancer and cancerous areas of "cancer in adenoma" showed similar levels of 26.3% (5/19) and 26.3%

Fig. 5. Results of dot blot hybridization with oligonucleotide probes specific for the normal sequence GGT of codon 12 (a), and mutated sequences TGT (b), AGT (c), CGT (d), GTT (e), GAT (f) and GCT (g). Cont., control; a, cancer; b, adenoma with severe atypia; c, adenoma with moderate or slight atypia; d, normal mucosa. Cases 1–6 correspond to those shown in Table II. Case 7 is a negative control (cancer in adenoma). Though case 3 showed a mutation only in the cancer area, cases 1, 2, 4 and 6 demonstrated positive results in areas of cancer and adenoma with severe atypia. In case 5, a mutation was detected in all areas other than normal mucosa. The nature of the mutation was homogeneous in each individual tumor.

(10/38), respectively. The lack of any appreciable difference between advanced cancers and "cancer in adenoma" lesions of the colorectum suggests that they occupy the same position from the standpoint of K-ras codon 12 point mutation.

While the incidence for VWDC was slightly higher than that for WDC according to our data, we can not conclude that grade of malignancy may be controlled by K-ras point mutation without considerable further analysis.

The notable increase in mutation between adenomas with moderate or slight atypia and severe atypia ($P<$ 0.05) is in line with earlier findings. According to Miyaki et al.[5] who analyzed each lesion as a whole from familial adenomatous polyposis (FAP) cases and reached a diagnosis using their criteria for dysplasia, 33 out of 35 cases with codon 12 or 13 mutations had a codon 12 mutation, with a remarkable increase during the transition from moderate dysplasia (11%) to moderate to severe or severe dysplasia (36%). However, the incidence decreased in intramucosal cancer (22%) and increased again in invasive cancer (40%). According to Ando et al.'s data[6] on tumors from FAP and non-FAP cases, total incidences for adenoma with mild atypia, adenoma with moderate atypia, adenoma with severe atypia, mucosal carcinoma and advanced cancer were 0% (0/17), 8.1% (3/37), 83.3% (15/18), 66.7% (2/3) and 26.0% (19/73), respectively. Although the reason why the incidence for adenoma with severe atypia was so high in the last author's study is unknown, a remarkable increase in incidence during a transition from low grade atypia to severe atypia was indicated. Thus, we conclude that K-ras point mutation does play an important role in early stages of colorectal tumorigenesis. Further, Miyaki et al.[5] indicated that K-ras point mutation also contributes to the malignancy grade of colorectal carcinomas, but we could not confirm this.

No mutation was observed in any part of three flat-type tumors examined. Similarly Fujimori et al.[7] could not detect any K-ras codon 12 point mutations in superficial early colorectal cancers of flat or depressed type. These data suggest that different pathways of colorectal tumorigenesis exist for the polypoid and flat types.

A GGT (Gly) to GAT (Asp) type mutation predominated in the present study (eight cases out of 10). Many other reports have also documented a high incidence of mutations resulting in coding for Asp: 80.0%

(4/5) by Shaw et al.,[8] 77.8% (7/9) by Salhab et al.,[4] 55.6% (5/9) by Bos et al.,[1] and 36.6% (11/30) by Vogelstein et al.[3] In Japan, Hayakumo et al.[9] similarly described a relatively high incidence (34.4%, 11/32) of mutation to Asp. However, Burmer and Loeb[10] reported that all mutations at the first pair of codon 12 caused a G-A transition, reflected in an amino acid change from Gly to Ser. Thus, there is no definitive shift. Whether the heterogeneity is based on racial or environmental difference or other factors is a subject requiring clarification.

The present experimental approach allowed clear demonstration that different atypical areas from the same tumor (cancer in adenoma) have a homogeneous mutation. Although 6 cases out of eight showed mutation to GAT, it should be noted that in two cases with different mutations from that to GAT the mutations were also homogeneous (one to TGT, one to GTT). According to Bos et al.,[1] five of 6 colorectal tumors with cancerous and adenomatous components also showed homogeneous mutation within the same tumor. These data provide genetic support for the adenoma-carcinoma sequence that was proposed on the basis of morphological data. No mutation was detected in normal mucosa, in line with most previous studies, while Burmer and Loeb[10] reported two cases of the K-ras codon 12 point mutation in normal mucosa adjacent to cancers. The question of possible contamination arises. They used histological enrichment and cell sorting in an sttempt to eliminate this problem, but the key point in this kind of examination is how accurately the target areas can be sampled. To ensure accuracy, we used hematoxylin-stained specimens and collected tiny tissue samples precisely from selected areas under a stereomicroscope with syringe needles. Adoption of this approach should facilitate a more profound understanding of tumorigenesis in various organs.

ACKNOWLEDGMENTS

REFERENCES

1) Bos, J. L., Fearon, E. R., Hamilton, S. R., Verlaan-de Vries, M., van Boom, J. H., van der Eb, A. J. and Vogelstein, B. Prevalence of ras gene mutations in human colorectal cancers. *Nature*, **327**, 293–297 (1987).

2) Forrester, K., Almoguera, C., Han, K., Grizzle, W. E. and Perucho, M. Detection of high incidence of K-ras oncogenes during human colon tumorigenesis. *Nature*, **327**, 298–303 (1987).

3) Vogelstein, B., Fearon, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., Leppert, M., Nakamura, Y., White, R., Smits, A. M. M. and Bos, J. L. Genetic alterations during colorectal-tumor development. *N. Engl. J. Med.*, **319**, 525–532 (1988).

4) Salhab, N., Jones, D. J., Bos, J. L., Kinsella, A. and Schofield, P. F. Detection of *ras* gene alterations and *ras* protein in colorectal cancer. *Dis. Colon Rectum*, **32**, 659–664 (1989).

5) Miyaki, M., Seki, M., Okamoto, M., Yamanaka, A., Maeda, Y., Tanaka, K., Kikuchi, R., Iwama, T., Ikeuchi, T., Tonomura, A., Nakamura, Y., White, R., Miki, Y., Utsunomiya, J. and Koike, M. Genetic changes and histological types in colorectal tumors from patients with familial adenomatous polyposis. *Cancer Res.*, **50**, 7166–7173 (1990).

6) Ando, M., Maruyama, M., Oto, M., Takemura, K., Endo, M. and Yuasa, Y. Higher frequency of point mutations in the c-K-*ras* 2 gene in human colorectal adenomas with severe atypia than in carcinomas. *Jpn. J. Cancer Res.*, **82**, 245–249 (1991).

7) Fujimori, T., Nagasako, K., Satonaka, K., Hirayama, D., Harada, M., Muratani, A., Tabata, T., Kitazawa, S., Ikehara, H. and Maeda, S. Expression of *ras* oncogene product (p-21) and point mutation of Ki-ras codon 12 in superficial early colorectal cancer. *I to Cho*, **27**, 111–117 (1992) (in Japanese).

8) Shaw, P., Tardy, S., Benito, E., Obrador, A. and Costa, J. Occurrence of K-*ras* and p53 mutations in primary colorectal tumors. *Oncogene*, **6**, 2121–2128 (1991).

9) Hayakumo, T., Azuma, T., Nakajima, M., Yasuda, K., Cho, E., Mukai, H., Mizuma, Y., Ashihara, T., Mizuno, S., Hirano, S., Ikeda, E., Kato, G., Tokuda, H., Takenaka, A., Izumi, H., Ikawa, O., Aoike, A. and Kawai, K. Prevalence of K-ras gene mutation in human colorectal cancers. *Gastroenterol. Jpn.*, **88**, 1539–1544 (1991) (in Japanese).

10) Burmer, G. C. and Loeb, L. A. Mutations in *KRAS2* oncogene during progressive stages of human colon carcinoma. *Proc. Natl. Acad. Sci. USA*, **86**, 2403–2407 (1989).

11) Hollstein, M., Sidransky, D., Vogelstein, B. and Harris, C. C. p53 mutations in human cancers. *Science*, **253**, 49–53 (1991).

12) Fearon, E. R., Cho, K. R., Nigro, J. M., Kern, S. E., Simons, J. W., Ruppert, J. M., Hamilton, S. R., Preisinger, A. C., Thomas, G., Kinzler, K. W. and Vogelstein, B. Identification of a chromosome 18q gene that is altered in human colorectal cancers. *Science*, **247**, 49–56 (1990).

13) Kinzler, K. W., Nilbert, M. C., Vogelstein, B., Bryan, T. M., Levy, D. B., Smith, K. J., Preisinger, A. C., Hamilton, S. R., Hedge, P., Markham, A., Carlson, M., Joslyn, G., Groden, J., White, R., Miki, Y., Miyoshi, Y., Nishisho, I. and Nakamura, Y. Identification of a gene located at

chromosome 5q21 that is mutated in colon cancers. *Science*, **251**, 1366–1370 (1991).

14) Kinzler, K. W., Nilbert, M. C., Su, L.-K., Vogelstein, B., Bryan, T. M., Levy, D. B., Smith, K. J., Preisinger, A. C., Hedge, P., McKechnie, D., Finniear, R., Markham, A., Groffen, J., Boguski, M. S., Altschul, S. F., Horii, A., Ando, H., Miyoshi, Y., Miki, Y., Nishisho, I. and Nakamura, Y. Identification of FAP locus genes from chromosome 5q21. *Science*, **253**, 661–665 (1991).

15) Nishisho, I., Nakamura, Y., Miyoshi, Y., Miki, Y., Ando, H., Horii, A., Koyama, K., Utsunomiya, J., Baba, S., Hedge, P., Markham, A, Krush, A. J., Peterson, G., Hamilton, S. R., Nilbert, M. C., Levy, D. B., Bryan, T. M., Preisinger, A. C., Smith, K. J., Su, L.-K., Kinzler, K. W. and Vogelstein, B. Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. *Science*, **253**, 665–669 (1991).

16) Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., Albertsen, H., Joslyn, G., Stevens, J., Spirio, L., Robertson, M., Sargeant, L., Krapcho, K., Wolff, E., Burt, R., Hughes, J. P., Warrington, J., McPherson, J., Wasmuth, J., Paslier, D. L., Abderrahim, H., Cohen, D., Leppert, M. and White, R. Identification and characterization of the familial adenomatous polyposis coli gene. *Cell*, **66**, 589–600 (1991).

17) Joslyn, G., Carlson, M., Thliveris, A., Albertsen, H., Gelbert, L., Samowitz, W., Groden, J., Stevens, J., Spirio, L., Robertson, M., Sargeant, L., Krapcho, K., Wolff, E., Burt, R., Hughes, J. P., Warrington, J., McPherson, J., Wasmuth, J., Paslier, D. L., Abderrahim, H., Cohen, D., Leppert, M. and White, R. Identification of deletion mutations and three new genes at the familial polyposis locus. *Cell*, **66**, 601–613 (1991).

18) Yanagisawa, A., Kato, Y., Ohtake, K., Kitagawa, T., Ohashi, K., Hori, M., Takagi, K. and Sugano, H. c-Ki-*ras* point mutations in ductectatic-type mucinous cystic neoplasms of the pancreas. *Jpn. J. Cancer Res.*, **82**, 1057–1060 (1991).

19) Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A. and Arheim, N. Enzymatic amplification of β-globulin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science*, **230**, 1350–1354 (1985).

20) Wright, D. K. and Manos, M. M. Sample preparation from paraffin-embedded tissues. *In* "PCR Protocols: A Guide to Methods and Applications," pp. 153–158 (1990). Academic Press, Inc., New York.

21) Verlaan-de Vries, M., Bogaard, M. E., van den Elst, H., van Boom, J. H. and van der Eb, A. J. and Bos, J. L. A dot-blot screening procedure for mutated *ras* oncogenes using synthetic oligonucleotides. *Gene*, **50**, 313–320 (1986).